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AUTHORITY

USABL ltr, 13 Sep 1971

TRANSLATION NO. 161

DATE: Sept 1968

DEPARTMENT OF THE ARMY
Fort Detrick
Frederick, Maryland

Zeitschr. fur Immunitatsforsch. bd. 73, Heft 1/2: 137-148, 1931.

Hober, F. - The bacteriophage-content of Chicken-droppings and the Influence of Feeding of Dysentery Bacteria on the Origin of bacteriophages

One of the rarest phenomena in the area of bacteriophage research is the occurrence of bacteriophage in animals which work quite strongly and occasionally apparently exclusively against human intestinal parasites, such as typhus, cholera, and dysentery-germs, with which they have never come in contact. Thus, for example are found dysentery-phages in crows (Haden), typhus phages in cattle, swine, etc. The chicken-dropping is recognized as one of the most productive sources of bacteriophages against all gram-negative bacteria (D'Herelle, Bail, Watanabe, Hoder and Suzuki, Klieneberger, etc.). However, up to now the questio- of the origin of the lysine has not been solved. Two possibilities arise (1) th autochthonous origin of the phages in the intestine and other organs of the animal concerned, and (2) the taking on the lysine from the surroundings, where they are always abundant. In sewage, tap water, ground water, etc.

New-born animals and foetuses are free of lysine (Vedrenne). In the meconium lysine was first detected on the 4th day (Suranji and Kramar). Van Loghem and Perelli found no phages in new-borne rabbits, and the animals remained phage-less when raised on ultrasterile food. Doerr found no phages in chicks raised sterile and unsterile up to the 7th day. Phages first occurred on the 15th day, and they were effective on Coli-germs of the intestines. Doerr described feeding with coli-bacteria which resulted in the occurrence of phages which worked not only on the coli-strain but also on other races. We, in contrast to Doerr, found, not only in sterilly-raised cocks but also in chicks (pullets) which had been fed on Bact. coli and various human-pathogenic intestinal germs throughout their lives, neither coli-phages nor typhus-coli-dysen-

tery-phages.

Borchardt isolated bacteriophages from feces of rabbits which had been fed various dysentery-strains after neutralizing of the stomach-acid. This showed that pancreas-ferment activated by the enterokinase is the "primum movens" for the lysate-formation.

Marcuse found likewise no phages in cocks, but he did find phages in a large percentage of grown hens and in all pigeons. He also leaves unanswered the question of the source of the lysine. Sonnenschein maintains that the supposed spontaneous origin of bacteriophages which has been maintained on many sides, is not at all demonstrable. He points out correctly the great range (spread) or phages, and assumes that the phage-carrying animals take on the lysine from their environment with food and water.

In the course of numerous experiments we had observed that at times the feces of chickens as well as of other animals were extraordinarily phage-rich, but then again at times they are phage-free, or at least contained no phage for the particular bacteria we were using at the time. We tried by means of systematic thoroughgoing research on the feces of chicks to establish how the phage-content is variable in animals under normal and pathologic conditions.

We posed the following questions: (1) Does the phage-content of the normal chicken intestinal tract remain constant under constant diet? (2) When an animal, whose feces does not contain a phage, for a particular bacterium, is fed that strain of bacterium, does the phase for that bacterium arise in the animal?

In our tests we used three young chickens, which came direct from the brooder-installation at the institute. Up to then they had not come in con-

tact with grown chickens nor had they been in the same room with grown chickens.

Hen 1 and Hen 2 were 4 weeks old and Hen 3 was 6 weeks old. The animals were placed, immediately on receipt, in separate, previously sterilised cages. In the stall were also found guinea pigs and rabbits, which also were likewise placed in cages, so that a direct transfer of phages to the chickens appeared quite out of the question. The hens themselves never came in direct contact with one another. During the time of the test the food was always the same for all three chickens, and consisted of commercial so-called chickenfeed (finely ground) and tap-water.

We extracted each day from each animal 5 gms of the freshest dropping we could find, and manipulated in in the following manner:

The feces was placed in a morter with 20 cc bouillon and ground up (pH 7.2) and was placed in a flask and allowed to stand at room temperature 24 hours. The next day the suspension was centrifuged and the clear liquid obtained was warmed 25 min. at 58°. This temperature suffices to kill the vegetative forms transferred to the feces in the bouillon or at least to diminish them so much that they do not interfere with the obtaining to pure culture of the germs to be investigated. As a rule a completely pure culture of the pathogen can be obtained. Occasionally the common Subtilis or other aerobic spore-bacilli increase greatly in addition to the gram-negative germs, but still not so greatly that they would interfere with the analysis of the test. The phages were not phased by the temperature of 58°. This method is simpler and quicker than the usual filtration-method.

The test of bacteriopheges followed in two stages.

Next direct smears to agar-plates were made (1 Oese of a 24 hour bouillon culture of the bacterium concerned plus an Oese of the feces-centrifugate). At the same time was undertaken an enriching of the phages present eventually in the centrifugate only in small numbers. To this purpose each 5 cm bouillon was innoculated with ½ cm of the centrifugate and by 1 Oese of a fresh bouillon culture of the bacterium to be tested, the tubelets after 24 hour incubation, regardless of whether or not they showed sediment, warmed 25 min. in water bath at 58°, and then tested for phage by smearing - 1 Oese of the heated bouillon plus 1 Oese of the fresh bacterium.

The second part of the test proved to be best. Repeatedly after the enrichment, which made possible the use of a larger amount of the original centrifugate, the phage was established as present, whereas direct smears did not show detectable phage.

We worked exclusively with dysentery-bacteria, with 22 different strains. The large number were chosen since it was important to us to find dysentery strains against which our hens after several weeks produced no phages, since it was only after feeding of such strains that we could obtain results which were to some degree significant.

In the first test we tried to establish the phage-content of the 3 hens. Each day we extracted 3-5 g of fresh feces and manipulated it in the manner described above. For four weeks, while the tests ran, the dict was constant, homogeneous. For brevity, we present the results only for Hen 2. (Table I). The feces of the other two hens gave similar results. We show in the table only positive v. negative occurrence, without showing the actual activity of the phages, and without indicating whether the results were obtained using direct smears or smears of enriched centrifugate.

Table I

Day	1	2	3	5	6	7	8	9	10	12	13	14	15	16
Strains (22)														
Hiss Meyer	p	p	n	n	n	n	n	p	n	n	n	p	p	n
Pseudodys. Leipzig H	p	n	n	p	p	n	n	n	p	p	n	n	p	n
Pseudodys. Leipzig A	n	p	n	p	n	n	n	n	n	p	n	n	n	n
Pseudodys. Leipzig D	n	n	n	p	n	n	n	p	p	n	p	n	p	n
Dysent. Tuch	p	p	n	n	n	n	n	n	n	n	n	p	p	n
Shiga Amsterdam	p	p	n	n	p	p	p	p	n	n	p	n	n	n
Hiss Pavia	p	n	n	n	n	n	p	n	n	n	n	n	n	p
Sonne Meyer	p	p	p	p	n	n	p	p	n	n	n	p	n	p
Flexner Meyer	n	n	n	p	n	n	n	n	n	n	p	n	n	n
Flexner basel	n	n	n	n	n	n	n	n	n	p	n	n	n	n
E. Kruse basel	p	p	n	p	n	p	p	p	n	p	n	p	p	p
Shiga R.-G. Amt.	p	p	n	p	p	p	p	p	p	p	n	p	p	p
Shiga Charkov	n	p	n	n	n	n	p	n	n	n	n	p	n	n
Flexner	n	n	n	p	n	n	n	n	p	n	n	n	n	n
Hiss	n	n	n	n	n	n	n	n	n	n	n	n	n	n
Schmitz	n	n	n	n	n	n	p	p	n	n	n	p	n	n
Coli comm.	n	n	n	n	n	n	n	n	p	n	n	n	n	n
E. Kruse	p	p	p	p	n	n	p	p	n	n	n	p	n	p
Coli sensible	p	p	n	n	n	p	p	n	n	p	p	n	p	n
Shiga Meyer	n	p	n	n	n	n	n	n	n	n	n	p	n	n
Hiss Amsterdam	n	n	n	n	n	n	n	n	n	n	n	n	n	n
Flexner Frankfurt	p	n	n	p	n	p	n	n	n	p	p	p	n	n

The results of the continuous test was, as the table shows, extraordinarily surprising. A comparison of the positive occurrences shows, that the phage-content of the single stools was also nowhere near constant; phage-rich days followed phage-poor days and vice versa. A second astonishing outcome was that during the course of the test occurred phages against almost all of the strains employed. The Hen poorest in phage was Hen 1, the richest was Hen 2. At the end of the test, the following strains had not been lysed:

Hen 1: Pseudodysentery Leipzig A, Pseudodysentery Leipzig D, Dysentery Tuch, Bact. Coli commune, Hiss Amsterdam and Hiss Pavia.

Hen 2: Hiss and Hiss Amsterdam.

Hen 3: Pseudodysentery Leipzig A, Hiss Pavia, Flexner basel and bact. coli commune.

We can also assume that against the named strains with a known probability were present no phages in the intestinal tract of the concerned animals.

Unfortunately the Hen 1 died shortly after the first test from unknown causes, so that we were able to pursue the experiment only with the other two hens.

In the next test we sought to find the sources of the varying occurrences of bacteriophages, and next used large and small amounts of feces, since we thought of the possibility of a hindrance-activity of the feces in itself.

The entire daily discharge of the hens taken and mixed up together, and two 'aliquots' taken, one of 1 g. and one of 15 g., these amounts being mixed each with bouillon, and manipulated in the same manner as described above.

Table II

	1g	15g		1g	15g
Hiss Meyer	n	n	Shiga R.-G.-Amt.	n	n
Pseudodys. Leipzig H	n	n	Shiga Charkov	n	n
Pseudodys. Leipzig A	n	n	Flexner	n	n
Pseudocys. Leipzig D	n	n	Hiss	n	n
Dys. Tuch	n	n	Schmitz	n	n
Shiga Amsterdam	p	p	Coli commune	n	n
Hiss Pavia	n	n	E. Kruse	p	p
Sonne Meyer	n	n	Coli sensible	p	p
Flexner Meyer	p	p	Shiga Meyer	p	p
Flexner Basel	n	n	Hiss Amsterdam	n	n
E. Kruse, Basel	n	n	Flexner Frankfurt	n	n

This table shows, that the phage-content in both amounts of feces was the same. Repetition of the test resulted in similar outcome, but one time the phage was more abundant in the smaller amount than in the larger amount of feces,

obviously the result of incomplete mixing of feces of varying phage-content.

In a further test we tested two discharges from Hen 2. The first was taken late in the morning the second early in the afternoon. After the first discharge, to prevent the possibility of mixing the two, the hen was placed in a fresh, sterile cage. (Table III).

The phage-content even in these two discharges is very variable. The first discharge contained only two, the second up to 7 phages. In this case also repeat-tests gave similar results. In no case did two successive stools contain the same lysine.

We conclude therefrom that the phages were probably discharged in batches, perhaps as in typhus-convalescence when the bacilli are still carried in the intestine after convalescence has begun.

Table III

	A	B		A	B
Kiss Meyer	n	p	Shiga Reichsges.-Amt.	n	n
Pseudodys. Leipzig H	p	p	Shiga Charkov	n	n
Pseudodys. Leipzig A	n	p	Flexner	n	p
Pseudodys. Leipzig D	n	p	Kiss	n	n
Dysent. Tuch	n	n	Schmitz	n	n
Shiga Amsterdam	n	n	Coli commune	n	n
Kiss Pavia	n	n	E. Krause	n	n
Sonne Meyer	n	n	Coli sensible	n	n
Flexner Meyer	n	p	Shiga Meyer	p	n
Flexner Basel	n	p	Kiss Amsterdam	n	n
E. Kruse Basel	n	n	Flexner Frankfurt	n	n

A is the morning discharge: 2.5 g.
B is the afternoon discharge: 3 g.

A repeated check of pH of the bouillon before use and after centrifuging of the manipulated feces indicated that there was no significant change of pH during

the standing 24-hours. Whereas the pH of the starting-bouillon was 7.2, that of the clear fluid after centrifuging was 7-7.1. It could not be possible, therefore, that change of pH was the cause of change of phage-content.

As we have already gathered from Test 1, there remained only a few strains against which during the 4-week duration of the test phages did not arise. We sought now to determine whether phages would arise against these particular strains if they were fed to the chickens.

In Hen 2 only phages against Hiss and Hiss Amsterdam had not arisen.

One Oese of a fresh agar culture of Hiss was mixed in a physiological saline, put with sterile broth, and fed to the animal. The Hen 2 fed on this mixture in preference to the chicken-feed which had been its exclusive diet.

A test of the feces before the infection showed numerous coli-bacilli and numerous gram positive, slender, often thread-like rods. After infection this flora remained unchanged. The animal remained healthy. We did not succeed in detecting in its feces any of the Hiss bacilli which we had fed to it.

Twenty-four hours after the infection the first feces was sampled, and manipulated in the same way as before. Neither this test, nor the several succeeding ones during the following 8-day period, showed any content of a phage against the Hiss bacilli.

On the 9th day the Hen 2 was again fed a fresh Hiss-culture. This time the animal sickened and developed bad diarrhea. The feces contained normal Coli and the other mentioned gram-positive rods and also occasionally paracoli-bacilli. This time also we were not able to detect Hiss-bacilli in the feces. The phage-content was enhanced astonishingly, so that of the 22 strains, 19 were lysed. However we were still not able to demonstrate the presence of phage

against Hiss-bacilli.

After 10 days, since by this time the chicken was discharging the usual feces, we again infected it, this time however with an Oese of Hiss Amsterdam.

The hen sickened, became paralysed in its extremities and could not support itself, and had diarrhea. Immediately after the infection we were able to detect the Hiss Amsterdam in the feces.

In the following two days occurred phages against a series of other germs, but none against Hiss Amsterdam. On the 22nd day a phage against Hiss Amsterdam did appear, which disappeared on the 23rd day, reappeared on the 24th, and finally disappeared. It was last seen on the 29th day. On the 30th day the hen died.

In the 4th, 5th and 6th tables we present merely the results of the tests using the strains against which, in the healthy animals, no phages had occurred up to the beginning of the feeding with the bacterial-strain. As a matter of fact, in all the tests, all 22 strains were actually tested.

Table IV

	1. Infection with Hiss									2. Infection with Hiss							1. Infection with Hiss Amsterdam									
Day	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26
Hiss	n	n	n	n	n	n	n	n	n	n	n	n	n	n	n	n	n	n	n	n	n	n	n	n	n	n
Hiss Amsterdam	n	n	n	n	n	n	n	n	n	n	n	n	n	n	n	n	n	n	n	n	n	p	n	p	n	n

Hen 3: In the continuing tests of feces from Hen 3 (pre-infection) no phages occurred against the following strains: Pseudodysentery Leipzig A, Hiss Pavia, Flexner Basel, and bact. coli commune.

The bacteriological tests of the normal feces of Hen 3 shows predominantly normal Coli, in addition to a few short, gram positive reds, and single gram

positive cocci.

In the first feeding test we infected Hen 3 with an Case of Pseudo-dysentery Leipzig A and Hiss Pavia. The feces-flora was not changed by the infection. Immediately after the feeding we were once able to detect dysentery-bacilli. The animal sickened with diarrhea. The tests for phages were, as in the previous tests, carried out with the largest possible amount of chicken feces.

On the first day after infection no phages occurred against the Leipzig A and Hiss Pavia. But on the 2nd day appeared a strong phage against Hiss Pavia. But on the 3rd day that phage had dwindled. The animal remained sick, had diarrhea, and was hardly able to move. Up to the 7th day it had just about held its own, and at part the feces were not as runny. The phage against Hiss Pavia had not shown up again, whereas that against the second germ, Pseudo-dysnetery Leipzig A had not shown up at all.

On the 7th day the feeding with both strains was repeated. The animals again got diarrhea and could not support themselves. (Lame in both legs).

On the 9th day a phage against Hiss Pavia showed up, but that again disappeared on the 10th day. On the 10th day appeared a phage against Flexner Basel, a strain which up to that time had not been fed. On the 11th day the Flexner Basel phage was also no longer detectable. On the 13th day phages against the two strains which had been fed showed up. At this time the animal partially recovered again and presented a fairly healthy aspect. The diarrhea disappeared and the stool was normal. Likewise the paralysis diminished.

On the 16th day suddenly occurred a powerful phage against the bact. coli commune, which had not been fed at all, and the Flexner basel phage reappeared.

We intended on the 16th day to feed Coli strain, since that was the only one against which no phage had shown up. But the coli phage appeared spontaneously that day just prior to the feeding of it. We carried out the feeding with coli the next day just as described above.

The animal ate the stuff readily; the feces remained normal.

Table V

	1. Infection with Pseudodys. Leipz. A + Hiss Pavia							2. Infection with Pseudodys. Leipz. A + Hiss Pavia							3. Infection with Bact. coli commune								
Day	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
Pseudodys. Leipzig A	n	n	n	n	n	n	n	n	n	n	n	n	p	n	n	n	n	n	n	n	n	n	n	n
Hiss Pavia	n	p	n	n	n	n	n	n	p	n	n	n	p	n	n	n	n	p	n	n	n	r.	n	n
Flexner Basel	n	n	n	n	n	n	n	n	n	p	n	n	n	n	n	p	n	n	n	n	n	n	n	n
E. coli commune	n	n	n	n	n	n	n	n	n	n	n	n	n	n	n	p	n	n	n	r	n	n	n	n

In spite of the feeding, which according to our previous conceptions should have led to an enrichment of the coli-phages in the intestinal tract of the animal, the coli-phage remained absent in our tests. Instead the lysin against Hiss Pavia occurred again.

On the 24th day the test was broken off.

To find out the influence of the feeding on the phages which already were present in the feces, we fed, in a later test, 1 Oese of the strain dysentery Tuch and the strain E. Kruse Basel to the Hen 3. On the day of the infection phages against the three strains were detectable in the feces. Even here one must assume that the addition of a large amount of the sensitizing germs must have led to an increase of the homologous bacteriophages in the intestinal tract of the hen. but it shows that the fluctuations in content of both phages was the same before and after the feeding. The enhancement of content of the homologous bacteria had no appreciable influence on the occur-

rence of the lysines. (Table VI).

From our tests we conclude that the phage content of the hens, which indeed under conditions naturally favorable to infection should have little opportunity to pick up true dysentery-bacilli, or gram negative human pathogens, has nothing to do with the presence of the homologous bacteria. It is a question of the lysines occurring in the intestinal tract as far as possible coli-phages, which develop a more or less strong activity to bacteria of the typhus, paratyphus, and dysentery groups (most of all the latter), and which generate obviously entirely irregularly (or were secreted). It is naturally impossible or at least very difficult to detect the sensitizing coli-strain in the feces of the chicken, since the phage-activity inhibits the culturing of the sensitizing germ on the plate or in the bouillon. The question as to the formation of the phages, whether they arise in the intestinal tract of the animal or human, or whether they are introduced secondarily from without and first increase in the intestine, we cannot say for sure. We consider it doubtful that phages can arise voluntarily in the intestine of the chicken upon feeding by strongly sensitizing bacteria.

Table VI

	Infection							
	Before infection				After infection			
Days	4	3	2	1	1	2	3	4
Dysent. Tuch	p	p	n	p	n	n	p	n
E. Kruse Basel	n	p	p	p	p	p	n	n

Summary

1. The phage-content of chicken feces is extremely variable and in each test exhibits a different form. The irregularity in the occurrence of the phages is so great that even two immediately successive discharges show a

different phage-content.

2. It is immaterial whether a large or small amount of feces is used. The results in both cases remain the same.

3. The diversity in conditions of successive tests is not the result of differences in pH of the bouillon because of the manipulation of the feces.

4. During the coarse of many tests one can be certain of finding phages in the feces of chickens against almost any kind of dysentery-germ. Many phages recur very irregularly and disappear rapidly. Others are very common and in almost every test can be found without difficulty.

5. The artificial infection of chickens with dysentery-bacteria does not lead to the arising of homologous phages. When the phages for certain dysentery bacteria do occur along with those bacteria, still the occurrence is so irregular that we cannot draw a causal-relationship or correlation of the two. We observed in two cases the first occurrence of a particular phage immediately preceding the feeding of the bacteria concerned. The phage, however, was again missing after the feeding of the bacteria.

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